

Partial characterization of the silk allergens in mulberry silk extract

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SUMMARY

Although there have been reports of allergic reactions such as asthma and hypersensitivity pneumonitis to silk allergens (especially in oriental countries), these allergens have not been characterized. We report an in vivo and in vitro study of subjects allergic to silk, as well as a partial characterization of the allergens involved. Forty-one subjects with a clinical history of silk allergy who had asthma, allergic rhinitis or both, and who had a positive intradermal skin test to mulberry silk extract, were evaluated in the study. Four asthmatic subjects with negative skin tests and RASTs to mulberry silk extract were used as controls. RAST specific IgE was present in 37/41 (90%) subjects. SDS-PAGE of mulberry silk extract demonstrated 13 protein bands of less than 21-95 kD. IgE which bound to protein bands was present in 13/32 (41%) sera, twelve of which bound to a single protein with a molecular weight between 35.1 and 50 kD. A pool made with the four control sera did not react with any protein band. The patients allergic to silk showed evidence of sensitivity to mulberry silk extract, both in vivo and in vitro, but sera from 19/32 (59%) subjects did not have antibodies reactive with the mulberry silk extract immunoblot protein bands. The low avidity of the IgE may be responsible for this discrepancy between in vivo and in vitro results.

Key words: Silk allergens - Mulberry silk - Partial characterization - *Bombyx mori*

INTRODUCTION

China and Japan dominate the world production of silk. Allergy to silk products is known as an occupational hazard in sericulture. Asthma and rhinoconjunctivitis occur among workers involved in several stages of the production.

In the manufacturing process, the silk thread is reeled off the cocoon. Threads which are severely disrupted and broken are known as silk waste and are used to fill bed quilts, clothes and mattresses. There are reports of asthma, allergic rhinitis and hypersensitivity pneumonitis caused by allergens associated with waste substances of mulberry silk (*Bombyx mori*) (1-6) rather than with wild silk (*Antheraea* spp.) (5). On the contrary, textile products of silk are practically nonallergic because of the processing they go through during manufacturing.

This study compared the results of skin test and in vitro specific IgE determination in a group of subjects with clinical symptoms of allergy to silk and immunologically characterized the involved mulberry silk allergens.

MATERIALS AND METHODS

Patient population

Forty-one subjects with asthma and/or allergic rhinitis who had a clinical history of silk allergy and a positive intradermal skin test to mulberry silk extract were studied; 21/41 (51%) had asthma, two had allergic rhinitis and the remaining 18 had both. All 41 subjects were seen at the Peking Union Medical College (PUMC) in Beijing, China. None worked in the silk industry.

There were 22 (54%) males and 19 (46%) females with ages ranging from four to 66 years (mean age: 26 years). Sera were obtained from all 41 subjects allergic to silk and from four nonallergic asthmatic control patients with negative skin tests to silk.

The serum samples were studied at the University of South Florida College of Medicine and Veterans Hospital, Tampa, Florida, U.S.A., in a mutual collaboration with the PUMC allergy research personnel.

Silk extract

The mulberry silk extract used in this study was prepared from silk cocoons at the PUMC. Mulberry silk cocoons were cut into pieces and defatted with petroleum ether for 4 hours and then air dried. Extraction was performed at 1:15 weight/volume (w/v) with 0.2 M ammonium bicarbonate, pH 9 at 4 °C for 3 days. The supernatant was isolated by filtration under vacuum and then sterilized with a 0.2-µm filter. The total protein content was measured by the bicinchoninic acid method (Pierce Chemical Co., Rockford, Illinois).

Skin testing

All subjects had been tested with the mulberry silk extract by the intradermal route at a 1:1,500 dilution (w/v) and each skin reaction, measured at 15 min, compared with the negative control (saline solution). Wheals were measured in the longest diameter (D) and the diameter perpendicular to it (d) to obtain the mean wheal diameter (MWD = D+d/2). If the negative control also produced a wheal and flare reaction, it was subtracted from the MWD elicited by the mulberry silk extract.

The intensity of the reaction was graded from 0 to 4+ according to the following criteria: -: no wheal; 1+: MWD between 5-10 mm; 2+: MWD between 10-15 mm; 3+: MWD >15 mm or with pseudopods and 4+: MWD >15 mm and general symptoms (urticaria and/or angioedema, cough or bronchospasm) or >25 mm.

Identification of IgE antibodies against mulberry silk extract

Mulberry silk extract proteins were adsorbed to wells of Immulon 4 removable strips (Dynatech Laboratories, Inc., Chantilly, Virginia). Each well received 1 µg of mulberry silk extract protein in 100 µl of 0.2 M of sodium carbonate/bicarbonate buffer, pH 9.2. The wells were incubated overnight at room temperature in a humid box and washed four times with 0.1 M phosphate buffer, pH 7.5, containing 1% Tween 20 (washing buffer). After adding 100 µl of undiluted serum to each well, the wells were incubated overnight and washed four times with washing buffer. One hundred microliters of rabbit ¹²⁵I-anti-human IgE (Sanofi Diagnostics Pasteur Inc., Chaska, MN) diluted to 1:2 in 0.1 M phosphate buffer, pH 7.5, containing 1% Tween 20 and 1% BSA was then added to all wells, which were incubated overnight, again washed and the counts per minute (cpm) were measured in a gamma counter. The results were expressed as the percentage of the total counts bound (%TCB).

SDS-PAGE of the extract

SDS-PAGE was carried out in an 8-cm long, 5-20% polyacrylamide gradient gel (Ready Gels, BIO-RAD

Laboratories, Richmond, CA) with the discontinuous buffer system of Laemmli (7). Twenty-five microliters of the mulberry silk antigenic extract containing 15 µg of protein was treated with 10% mercaptoethanol, boiled for ten minutes and applied in a 4% polyacrylamide stacking gel. A set of molecular weight markers (Pre-stained SDS-PAGE Standards, low range, BIO-RAD Laboratories) was loaded with the silk extract. Electrophoresis was carried out in a Mini Protean II Dual Slab Cell (BIO-RAD Laboratories, Richmond, CA) at 200 V until the tracing dye reached the bottom of the gel.

After electrophoresis, the gels were either stained with silver nitrate (Silver Stain Kit, BIO-RAD Laboratories, Richmond, CA) or, alternatively, the proteins were immunoblotted.

Western blot

Transfer of the proteins to a 0.45-µm nitrocellulose membrane (BIO-RAD Laboratories, Richmond, CA), according to the method of Towbin *et al.* (8), was carried out at 150 mA constant power for 3 hours in a Mini-TransBlot Cell (BIO-RAD Laboratories, Richmond, CA). After the transfer, the nitrocellulose was blocked overnight with phosphate buffer, pH 7.5, containing 1% Tween 20 and 3% BSA to saturate the unbound sites.

The immunoblot was performed with 32 of the 41 sera from patients allergic to silk. Four of the nine remaining sera had a negative RAST and for five of them we did not have enough volume to perform the immunoblot.

Based on specific IgE levels, the sera were diluted to 1:2, 1:4 or 1:8 in 0.1M phosphate buffer, pH 7.5, containing 1% Tween 20 and 1% BSA, and were incubated overnight with the blotted membranes, at room temperature and with continuous shaking. The membranes were washed four times with 0.1 M phosphate buffer, pH 7.5, containing 1% Tween 20, and then incubated with rabbit ¹²⁵I-anti-human IgE (Sanofi Diagnostics Pasteur Inc., Chaska, MN) diluted to 1:10 in diluent buffer overnight at room temperature and with continuous shaking. After washing, the blots were exposed to an x-ray film (Kodak X-Omat, Sigma Chemical Co., St. Louis, MO) for 3, 7 or 25 days at -80 °C. Nonspecific binding of anti-IgE was evaluated with a serum pool prepared from the four asthmatic patients with negative skin test to the mulberry silk extract.

Avidity of the specific IgE

An analogous assay to that for specific IgE determination was performed with two pools of sera, each at two-fold dilutions, to a final dilution of 1:64. Pool 1 was made from 10 of the 13 sera that, in the Western blot, had presented bands recognized by the specific IgE. Pool 2 was made from eight of the 19 sera that, in the Western blot, did not present bands recognized by the specific IgE.

Table 1

Distribution of positive reactions in intradermal testing.

Degree of positive reaction	#	%
+	3	7.3
++	11	26.9
+++	21	51.2
++++	6	14.6

RESULTS

Skin test

All 41 patients were positive when skin tested with the mulberry silk extract (Table 1). All four controls had a negative skin test with the same extract.

Determination of specific IgE antibodies

The cutoff level (4 times the value for the negative pool) was 0.435% TCB (total counts bound). Thirty-seven of 41 (90%) of the patients allergic to silk had specific IgE to the mulberry silk extract and 11/41 (27%) had values exceeding 50% of TCB (Table 2). There was no significant correlation between specific IgE and the skin test ($r = 0.2$, $p = 0.2047$) results.

Table 2

Specific IgE results.

% TCB	#	%
<0.435	4	9.7
0.435-1	2	4.9
1-20	11	26.9
20-50	13	31.7
>50	11	26.8

TCB: total counts bound.

Avidity of the specific IgE to mulberry silk extract

The specific IgE determination for sera pools 1 and 2 shows that even the %TCB for the undiluted pools is similar (54.68 and 54.12%, respectively); the %TCB for pool 2 decreases beyond the 1:2 dilution, while for pool 1, it remains constant until 1:8 dilution (Fig. 1).

SDS-PAGE

Although there were considerable differences in intensity of silver nitrate stained bands, thirteen bands were demonstrated for the silk extract with a molecular weight range from less than 21.9 to 97.2 kD (Figure 2, lane 2). Major staining appeared for silk bands with a molecular weight of less than 21.9 kD, with the last three bands having a molecular weight of less than 14 kD. The three low molecular weight bands are probably glycoproteins, as determined by the yellow color that was present when they were stained with silver nitrate.

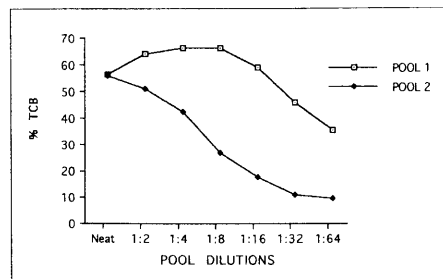


Fig. 1. Assay for the avidity of the specific IgE to mulberry silk extract allergens. Pool 1 contains IgE with higher avidity than pool 2.

Immunoblotting

Autoradiographs of the immunoblot analysis of the silk extract performed with 32 allergic patients' sera revealed, within one week of exposure, protein bands recognized by the specific IgE in 13 of the 32 (41%) sera. The remaining 19 sera were negative even after an exposure as long as 25 days.

For 12 of the 13 (92%) sera, specific IgE reacted to a protein band with a molecular weight between 35.1 and 50 kD (Figure 3, lanes 1-8; Figure 4, lanes 2-5). Nine of the 13 sera (69%) had specific IgE to one or two bands, both with molecular weights less than 21.9 kD (Figure 4, lanes 2-6; Figure 3, lanes 2, 5, 7 and 8). Nonspecific binding of IgE was found for the pool of sera used as a negative control (Figure 4, lane 1).

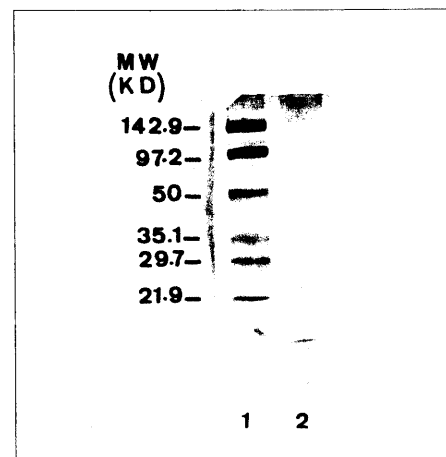


Fig. 2. SDS-PAGE of the mulberry silk extract, stained with silver nitrate. Lane 1: molecular weight standards; lane 2: mulberry silk extract (20 µg).

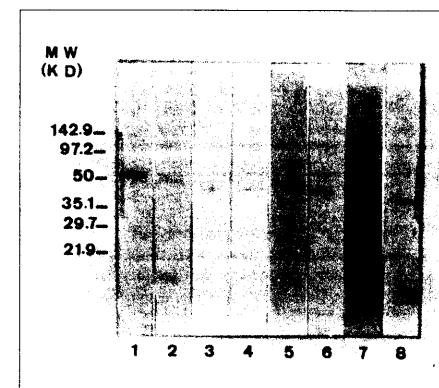


Fig. 3. SDS-PAGE/Western blot using the sera of eight allergic patients. Each lane represents a different serum.

DISCUSSION

Silk is divided into two groups: mulberry silk, produced by *Bombyx mori*, and wild silk, produced by *Anteraea* spp. Allergy to silk has been found to be associated with mulberry silk rather than wild silk (5). Silk thread consists of two filaments of fibroin glued together with a cementing substance named sericin. The allergenicity of both substances is unknown (5, 6).

In this study, skin testing and RAST performed with a mulberry silk extract was found to correlate with the presence of clinical symptoms in a group of 41 subjects allergic to silk waste. Percentages of positive results were similar to those reported by Zaoming et al. in a

sample of 64 children with asthma using a mulberry silk waste extract to establish sensitization by skin testing (1).

Autoradiographs of the immunoblots of the mulberry silk extract using 32 sera from silk allergic patients demonstrated protein bands which reacted with specific IgE in 13 (41%) of these sera.

The non-recognition of protein bands on the immunoblot by the specific IgE present in 19 of 32 (59%) of the sera could be due to the presence of low-avidity IgE antibodies to mulberry silk allergens. This is suggested by the rapid decrease of the RAST values found for the pool of sera made from 10 of them, parallel to an increase in their dilution.

For the 13 immunoblot-positive sera, while there is a variation in the bands recognized by the specific IgE, 12 of the 13 recognized a single protein band with a molecular weight between 35.1 and 50 kD. This protein seems to be the main mulberry silk allergen.

Further studies are needed to finally define the main silk allergens, studies which should be focused on the characterization of the main proteins in the silk, fibroin and sericin, of *Bombyx mori* cocoons.

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RESUMEN

Aunque existe evidencia de reacciones alérgicas del tipo del asma bronquial o la neumonitis por hipersensibilidad a los alérgenos de la seda, éstas no han sido bien caracterizadas. Con este estudio in vivo e in vitro de pacientes alérgicos a la seda producida por *Bombyx mori* se pretende obtener su caracterización parcial. Estudiamos 41 pacientes con rinitis alérgica y/o asma bronquial con historia clínica de alergia a la seda y prueba cutánea positiva al extracto preparado por PUMC (Peking University Medical College). Como controles utilizamos 4 sujetos asmáticos no alérgicos. Realizamos determinaciones de IgE específica, que fue positiva en el 90% de los pacientes, no encontrando correlación significativa entre la prueba cutánea y el valor de IgE específica. En el SDS-PAGE del extracto de seda objetivamos 13 bandas proteicas con un PM entre 21.9 y 97.2 kD, una banda intensa por debajo de los 21.9 kD y las tres últimas bandas con un PM <14 kD, posiblemente glucoproteínas. En el 41% de los casos se demostraron bandas proteicas que reaccionaron con la IgE específica en el Immunoblot, siendo en el 92% de los casos entre 35.1 y 50 kD; posiblemente sea en esta banda proteica donde se localice el alérgeno principal. La falta de reconocimiento de bandas proteicas por IgE específica en el 59% restante de los sueros podría ser explicada por la presencia de anticuerpos IgE de baja avidez por los alérgenos de la seda debido a la rápida disminución de los valores encontrados en el RAST en dicho pool de sueros, paralelamente a un incremento en su dilución.

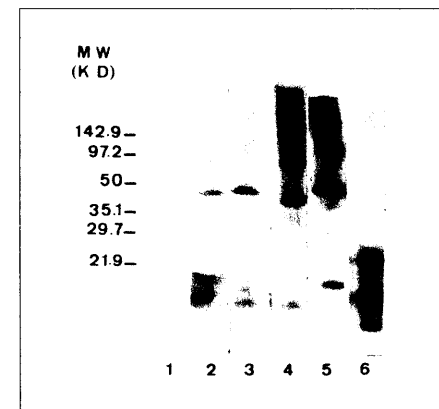


Fig. 4. SDS-PAGE/Western blot using the sera of 5 allergic patients (lanes 2 to 6) and a negative pool of sera to silk (lane 1).

Palabras clave: Alergenos seda - *Bombix mori* - Caracterización parcial - Aidez IgE

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